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Biochemistry

Structure of endogenous murine leukemia virus DNA in mouse genomes

(Southern blotting method/retroviruses/molecular cloning/ecotropic-specific probes)

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ABSTRACT **By using molecularly cloned ecotropic AKR murine leukemia virus (MuLV) DNA, a 400-base-pair ecotropic type-specific segment in the *env* region has been identified. This DNA segment and other defined viral subgenomic fragments have been used as ^{32}P-labeled probes to identify and analyze the structure of integrated ecotropic viral DNA sequences in uninfected mouse genomes. Those mice from which endogenous ecotropic MuLV of the AKR type have been isolated contained at least one virtually complete linear copy of the viral genome. Strains from which ecotropic MuLV has not been isolated lacked ecotropic-specific sequences. All inbred mouse strains tested also contained MuLV DNAs of genomic length whose restriction endonuclease digestion pattern was characteristic of xenotropic viruses.**

Normal mice contain endogenous murine leukemia virus (MuLV) sequences (1, 2) and MuLV expression follows Mendelian segregation in many strains (3, 4). Endogenous MuLVs have been divided into three related classes on the basis of their host range, which is determined by the viral envelope glycoprotein: ecotropic, which infect only murine cells; xenotropic, which infect primarily heterologous cells; and amphotropic, which replicate efficiently in cells of both types (5–7). Ecotropic MuLVs, which include the prototype Gross-AKR virus and represent the best-characterized class, are isolated with variable frequency from different mouse strains. In some mice, such as AKR, C58, C3H/Fg, PL, and *Mus musculus molossinus* (a wild Asian mouse), high levels of ecotropic MuLV are found uniformly among weanling mice, and this virus can be easily activated from early embryo cells by halogenated pyrimidines (high-ecotropic strains) (refs. 4 and 8; unpublished observations). In others, which include BALB/c, DBA, C3H/He, A/He, and NZW, ecotropic MuLV is isolated less frequently and is inefficiently activated from their cells (low-ecotropic strains) (9). In a third group, represented by NIH Swiss, C57L, 129, and NZB, ecotropic MuLV of the AKR type has not been isolated (nonecotropic strains).

Molecular hybridization of cell DNA from these mouse strains has shown a strong correlation between the number of complete copies of viral DNA of the AKR type and the frequency of ecotropic virus isolation, but the structure of the viral DNA has not been elucidated (2). High- and low-ecotropic strains contain multiple or single copies, respectively; nonecotropic strains lack a portion of the viral genome. All mice, however, contain multiple copies of sequences that crossreact with most of the AKR viral genome. Ecotropic virus-inducing loci of AKR (*Akv-1* and *Akv-2*), BALB/c, C57BL, and C3H/He have been shown to contain at least some viral DNA sequences (10–14).

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In the present study we used the Southern blotting technique (15) in conjunction with viral probes from defined regions of molecularly cloned ecotropic viral DNA (16) to demonstrate that endogenous ecotropic viral genomes of high- and low-virus strains are present as virtually complete linear copies of viral DNA and that some nonecotropic strains lack ecotropic-specific sequences from the *env* gene region.

MATERIALS AND METHODS

Cell DNAs and Viruses. Bulk cell DNAs were isolated as described (17). DNAs from *M. m. molossinus*, *M. m. castaneus*, *M. poschiavinus*, *M. caroli*, *M. dunii*, *M. cervicolor*, *M. cookii*, and *M. pahari* were obtained from cultured tail cells (18) established from mice generously provided by M. Potter (National Institutes of Health, A. Gropp (Klinikumder Medizinischen Hochschule Lübeck, Lubeck, West Germany), and T. C. Tsu (M. D. Anderson). Mink DNA was from Mv1Lu cells (ATCC line CCL64). DNAs from NZB-Q (19), SC-1 (20), and 129 were from established embryo cell lines. Inbred mouse strains were obtained from either The Jackson Laboratory (J) or the National Institutes of Health animal facility (N), except that C3H/Fg was obtained from E. Boyse (Memorial Sloan-Kettering, New York). For C3H/HeN, "+" and "−" refer to the presence and absence, respectively, of spontaneous expression of the mouse mammary tumor virus. NIH/N is an inbred NIH Swiss mouse (NFS). DNAs from 129/J and the other inbred mice were isolated from embryos. Most of the viruses in Fig. 2 have been described (7, 21, 22) with the exception of NFS-X and Mol-X, which are xenotropic MuLVs isolated from NFS and *M. m. molossinus*, respectively. Mol-X MuLV was isolated from tail cultures, and 623 MuLV was isolated from NIH 3T3 cells transfected with AKR MuLV DNA clone 623 (16). Other viruses were the generous gifts of J. W. Hartley (National Institutes of Health) and M. B. Gardner (University of Southern California).

DNA Probes. The viral probes used were derived from molecularly cloned integrated AKR MuLV DNA (clone 623) (16). Subgenomic viral fragments were isolated by preparative gel electrophoresis or by subcloning in pBR322. The cloned viral long terminal repeat (LTR) probe contained 1.4 and 0.6 kilobase pairs (kbp) of NIH 3T3 cell sequences to the left and right, respectively, of the LTR. The viral specific nature of the 8.2- and 7.6-kbp *Pst* I bands and the 1.4- and 1.5-kbp *Kpn* I bands visualized with the LTR probe was verified by prehybridization with unlabeled cloned 8.2-kbp *Pst* I viral DNA.

Abbreviations: MuLV, murine leukemia virus; LTR, long terminal repeat; kbp, kilobase pair(s).

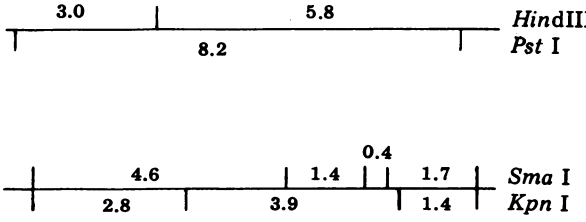

FIG. 1. Restriction endonuclease map of 8.8-kbp linear ecotropic AKR MuLV DNA. Numbers refer to the length (in kbp) of each internal restriction endonuclease fragment. *Eco*RI does not cleave the viral DNA.

RESULTS

Determination of an Ecotropic-Specific Region of Viral DNA. To analyze the viral sequences in cell DNA, it was necessary to have specific viral probes and to derive restriction endonuclease maps of known viral DNAs. We have recently developed maps for various ecotropic, xenotropic, and amphotropic MuLV DNAs (refs. 16 and 23; unpublished data). Ecotropic viruses isolated from many inbred mouse strains and from *M. m. molossinus* were found to have essentially identical maps. The studies reported here emphasize the structure of ecotropic viral DNA sequences, and the sites in the 8.8-kbp ecotropic AKR MuLV DNA for the restriction endonucleases used in this paper are shown in Fig. 1. *Eco*RI did not cleave most ecotropic viral DNAs, although it did cleave an ecotropic MuLV isolated from C3H/Fg and all amphotropic and xenotropic MuLVs tested. *Pst* I cleaved once within the 0.6-kbp LTR of unintegrated and integrated ecotropic MuLV. *Pst* I digestion therefore generated an 8.2-kbp internal viral fragment. *Kpn* I and *Sma* I were also useful for assessing internal viral fragments because they each cleaved the viral DNA several times, including once within each LTR (16).

In addition to using defined probes from different regions of the viral DNA, we analyzed the *env* region of the viral DNA for sequences that would hybridize selectively with ecotropic MuLVs because previous data has suggested that the *env* gene product contains host range specific determinants (24–26). A 0.4-kbp *Sma* I fragment located in this region 1.8–2.2 kbp from the 3′ end of the viral DNA (Fig. 1) was found to hybridize only with ecotropic MuLV DNAs (Fig. 2*A*). The specificity of this probe was also shown for *Eco*RI-digested cellular DNA. In contrast to results with a probe which contained all viral sequences (see Fig. 3*A*, lane k), the ecotropic-specific probe did not hybridize with DNA from uninfected NIH 3T3 cells (a nonecotropic strain), but it did show multiple copies in cells infected with ecotropic MuLV (Fig. 2*B*). The ecotropic virus inducing loci *Akv-1* and *Akv-2* of AKR/N have recently been localized to two large (>20 kbp) *Eco*RI fragments (14). These fragments, as well as a 10.5-kbp viral band, which presumably represents the recently described *Akv-3* (30), are seen in Fig. 2*B*. It is interesting to note that the lower of the two large *Eco*RI bands (*Akv-2*) is missing in AKR/J DNA, but it contains an additional smaller *Eco*RI viral fragment. The low-ecotropic BALB/c mouse contains a single viral band. These results correlate with those previously obtained by DNA reassociation kinetic analysis (2, 11, 13).

Structure of Viral DNA in Uninfected Cells. Using the ecotropic-specific sequences and other defined subgenomic fragments as probes, we have analyzed the viral DNA structure in a number of uninfected cells. Hybridization of *Eco*RI-digested cell DNA to a probe containing all AKR MuLV sequences (Fig. 3*A*) gave 10 or more bands in many strains of *M. musculus* and in *M. m. castaneus* and *M. poschiavinus*. The wild Asian mouse *M. m. molossinus* (lane h) contained the greatest number of viral DNA bands, 30 to 50. All mice contained some *Eco*RI bands that were shorter than genome length (8.8 kbp), suggesting either the presence of viral *Eco*RI sites in these molecules or of viral DNAs with deletions. Rat DNA contained significantly fewer bands than did *M. musculus*, but it contained more bands than *M. caroli* and mink; *M. dunni*, *M. cervicolor*, *M. cookii*, and *M. pahari* were almost negative for these sequences (Fig. 3*A*, lanes o–q; Fig. 4*A*, lanes n–q). Hybridization of the DNAs to a probe specific for the left half of the viral DNA (the 4.6-kbp *Sma* I fragment shown in Fig. 1) gave results (Fig. 3*B*) that were similar to those obtained with the full probe, indicating that the *M. musculus* strains all contained multiple copies of sequences highly related to this probe. By contrast, hybridization with the ecotropic-specific

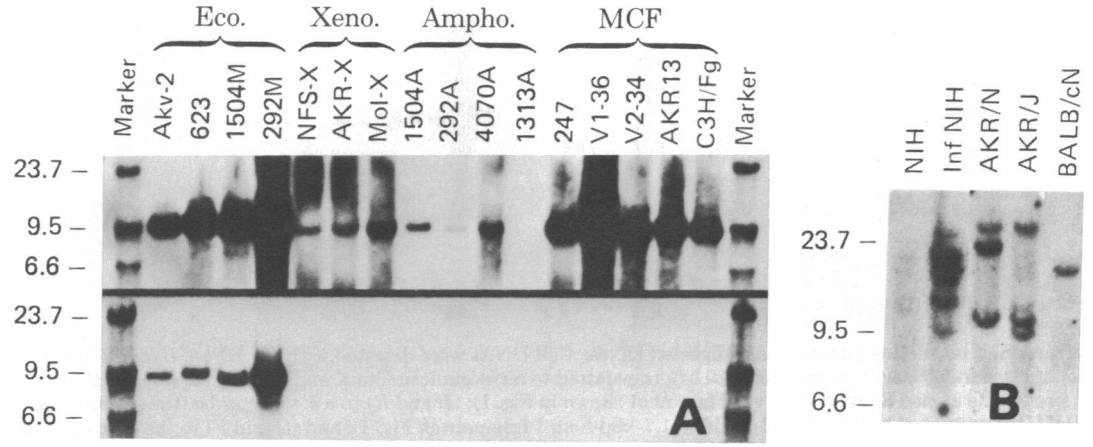

FIG. 2. Characterization of ecotropic AKR MuLV type-specific sequences. (*A*) Unintegrated proviral DNAs were isolated by the Hirt procedure 16–18 hr after infection of permissive cells (27). DNAs [5–7 μg of each for ecotropic viruses (Eco.), 15–20 μg of each for xenotropic (Xeno.) and amphotropic (Ampho.) viruses, and 10–12 μg of each for MCF viruses (21, 28)] were electrophoresed in horizontal 0.7% agarose gels (35 V; 16 hr), transferred to nitrocellulose filters (15), and hybridized with a ^{32}P-labeled nick-translated (29) 5′ viral DNA probe (although it contains 0.1 kbp of LTR sequence, the gel-purified 4.6-kbp *Sma* I fragment in Fig. 1 does not detect LTR sequences in the DNAs under these conditions of exposure) in top part of *A* or the ecotropic-specific probe (the cloned 0.4-kbp *Sma* I fragment in Fig. 1) bottom part of *A*. *Hin*dIII-digested ^{32}P-labeled λ DNA was used as marker. The numbers refer to the length (in kbp) of the marker DNA. The 5′ probe hybridized to the DNAs of viruses from all four groups, whereas the ecotropic-specific probe hybridized only to the ecotropic viruses. (*B*) *Eco*RI-digested high molecular weight cell DNAs (10–12 μg) were electrophoresed in 0.5% agarose gels, transferred to a nitrocellulose filter, and hybridized to the ^{32}P-labeled ecotropic-specific probe.

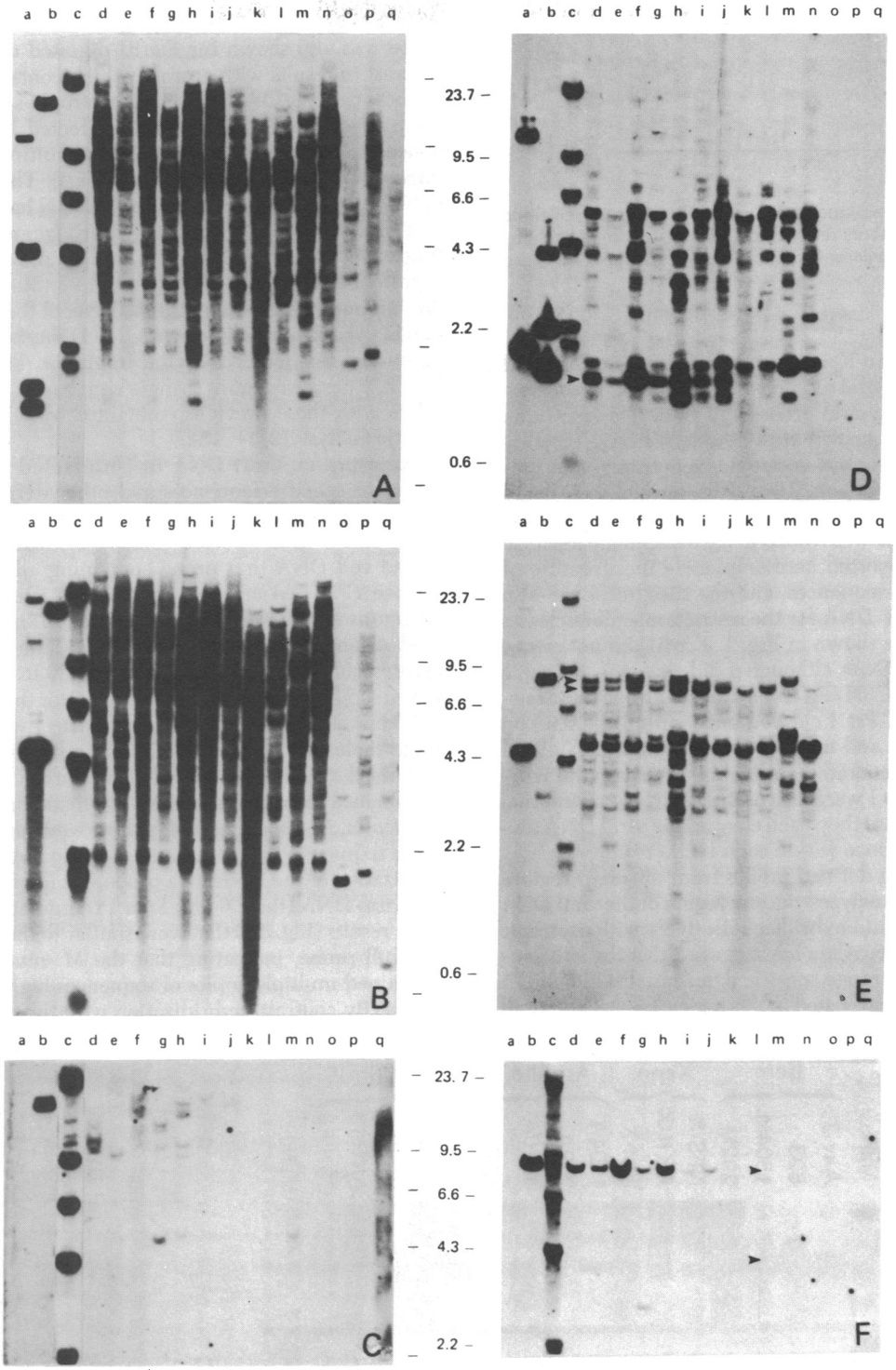

FIG. 3. Hybridization of restriction endonuclease-digested DNAs. Cell DNAs were digested with *Eco*RI (*A*, *B*, and *C*), *Kpn* I (*D*), or *Pst* I (*E* and *F*), electrophoresed in 0.5% agarose gels (35 V; 20 hr), transferred to nitrocellulose filters, and hybridized: (*A*) to ^{32}P-labeled full-length AKR MuLV DNA probe (the cloned 8.2-kbp *Pst* I viral fragment shown in Fig. 1); (*B* and *E*) to a 5′ viral probe (the gel-purified 4.6-kbp *Sma* I fragment in Fig. 1); (*D*) to a 3′ viral probe (the gel-purified 1.7-kbp *Sma* I fragment in Fig. 1); and (*C* and *F*) to the ecotropic-specific probe. Lanes: a, *Sma* I-digested clone 623 DNA; b, clone 623 DNA digested with *Eco*RI in *A–C*, with *Kpn* I in *D*, and with *Pst* I in *E* and *F*; c, *Hin*dIII-digested ^{32}P-labeled λ DNA; d, AKR/J; e, C58/J; f, C3H/Fg; g, PL/J; h, *M. m. molossinus;* i, BALB/cN; j, DBA/2N; k, NIH/N; l, 129/J; m, *M. m. castaneus;* n, *M. poschiavirus;* o, *M. caroli;* p, rat; q, mink. In *F*, lane m, arrowheads indicate the location of poorly hybridizing bands. The numbers represent the length (in kbp) of the marker DNAs.

probe (Fig. 3*C*) gave fewer bands in all mouse strains and distinguished among the high-ecotropic (lanes d–h, multiple bands), low-ecotropic (lanes i and j, single band), and nonecotropic (lanes k and l, no band) inbred strains. *M. m. castaneus* gave three faint bands (Fig. 3 *C*, lane m, and *F*, lane m, arrowheads), and cells from this wild mouse strain have consistently yielded ecotropic MuLV with a restriction endonuclease pattern that differs significantly from that obtained for AKR MuLV DNA (unpublished observations).

Kpn I cleaves viral DNAs from all classes multiple times,

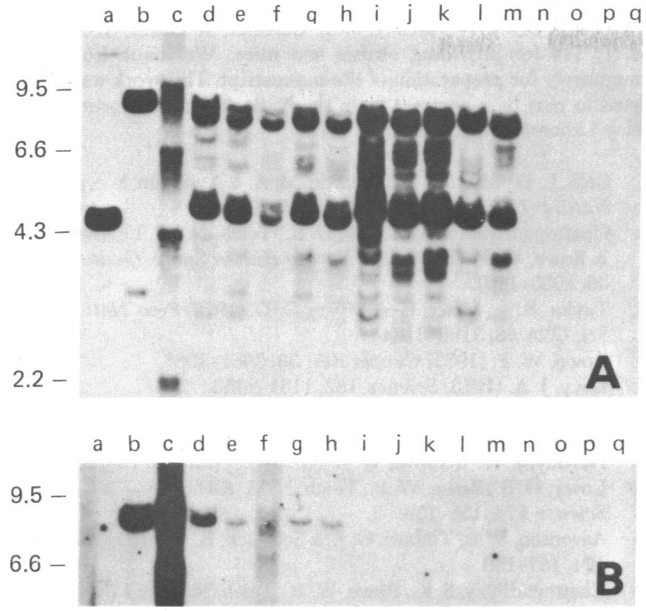

FIG. 4. Hybridization of *Pst* I-digested cell DNAs from various mouse strains. Cell DNAs were electrophoresed, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled 5′ probe (the 4.6-kbp *Sma* I fragment) in *A* or with the ecotropic-specific probe in *B*. Lanes: a, *Sma* I-digested clone 623; b, *Pst* I-digested clone 623; d–h and n–q, from embryos (d, AKR/J; e, A/HeN; f, NZW/N; g, C3H/HeN$^-$; h, C3H/HeN$^+$; n, *M. dunii*, o, *M. cervicolar;* p, *M. cookii;* q, *M. pahari);* i–m, from cell lines (i, C57L; j, NZB/N; k, NZB-Q; l, SC-1; m, 129). Marker DNAs were as in Fig. 3.

including once near the right-hand end of each LTR, and it is useful for the analysis of internal viral fragments. Hybridization of *Kpn* I-digested cell DNA to a probe specific for the 3′ end of the viral DNA including the LTR (the 1.7-kbp *Sma* I fragment in Fig. 1) revealed a 1.4-kbp cleavage product (Fig. 3*D*, arrowhead) in high- and low-ecotropic strains (with the exception of *M. m. castaneus*) which corresponded to the 1.4-kbp *Kpn* I-cleavage product at the 3′ end of AKR MuLV DNA (see Fig. 1) and was not found in the nonecotropic strains. The 3.9-kbp *Kpn* I band also was specific for cells with ecotropic viral genomes, if the hybridization was performed with the ecotropic-specific probe (data not shown). The 1.5-kbp *Kpn* I band, present in all the mouse strains, corresponded to a fragment mapped in the 3′ region of an AKR xenotropic MuLV. As noted above, *Pst* I digestion of AKR MuLV DNA, which cleaved less than 50 kbp from the left-hand end of each LTR, generated an internal 8.2-kbp fragment. The viral DNA from a xenotropic MuLV isolated from *M. m. molossinus* also contained only this site, but xenotropic MuLV isolated from several strains contained an additional *Pst* I site about 1.2 kbp from the 3′ end of the viral DNA, thus generating a 7.6-kbp internal xenotropic viral fragment (and one 0.6 kbp).

Compared with the hybridization pattern obtained when the 5′ probe was hybridized to *Eco*RI-digested DNAs (Fig. 3*B*), the *Pst* I pattern of hybridization with this probe was greatly simplified (Fig. 3*E*), which suggested that most of the prominent *Pst* I bands represented internal viral fragments. Note, for example, that the *Pst* I-digested DNAs lacked prominent fragments larger than 8.2 kbp. Similar results were seen with the full-length probe, except that a prominent 0.6-kbp band which corresponded to the *Pst* I band of this size in the xenotropic MuLVs noted above was also seen (data not shown). In view of the *Pst* I-cleavage products obtained with MuLV DNA, it was interesting that *Pst* I digestion of the cell DNAs revealed an 8.2-kbp band in those mice that contained endogenous ecotropic genomes and 7.6-kbp band in all *M. musculus* strains (Fig. 3*E*, arrowheads).

The presence of the 8.2- and 7.6-kbp bands suggested that these fragments represented portions of complete linear copies of ecotropic and xenotropic viral DNAs, respectively. Results consistent with this hypothesis have been obtained. We have previously determined that *Hin*dIII cleaves ecotropic viral DNA but not xenotropic (unpublished data). The presence and absence of *Hin*dIII sites in the 8.2- and 7.6-kbp bands, respectively, was confirmed by *Pst* I/*Hin*dIII digestion of representative DNAs (Fig. 5). In addition, the ecotropic-specific probe hybridized to the 8.2-kbp band but not to the 7.6-kbp band (Figs. 3*F* and 4*B*).

Although *Eco*RI digestion showed multiple ecotropic specific bands (Fig. 3*C*) among the high-ecotropic strains, the only ecotropic-specific band for *Pst* I-digested AKR and C58 was located at 8.2 kbp, and the 8.2-kbp band was the most prominent one for C3H/Fg, PL, and *M. m. molossinus*. The low-ecotropic mice all contained the ecotropic-specific 8.2-kbp *Pst* I band (Fig. 3*F*, lanes i and j; Fig. 4*B*, lanes e–h). As anticipated, the nonecotropic mice NFS/N, 129/J, and C57BL did not contain an ecotropic-specific *Pst* I band, although NZB, SC-1, and a 129 mouse cell line (in contrast to the 129/J embryo DNA) apparently contained poorly matched sequences that cross-reacted with the ecotropic-specific probe (Figs. 3*F* and 4*B*). The *env* gene product (the viral gp70) contains group-specific and type-specific determinants (24–26); the slight degree of hybridization of the ecotropic-specific probe to DNA from some nonecotropic strains suggests either that even some sequences in this probe may not be entirely type-specific or that these strains may contain some ecotropic sequences. A probe specific for the LTR also hybridized to the 8.2- and 7.6-kbp *Pst* I viral DNA bands, as well as to the 1.4- and 1.5-kbp *Kpn* I bands shown in Fig. 3*D* (data not shown). Radioactively labeled 1.4- and 1.7-kbp *Sma* I viral fragments (see Fig. 1) also yielded 8.2- and 7.6-kbp *Pst* I fragments (data not shown), which verified that these bands contained sequences from these regions of the viral DNA.

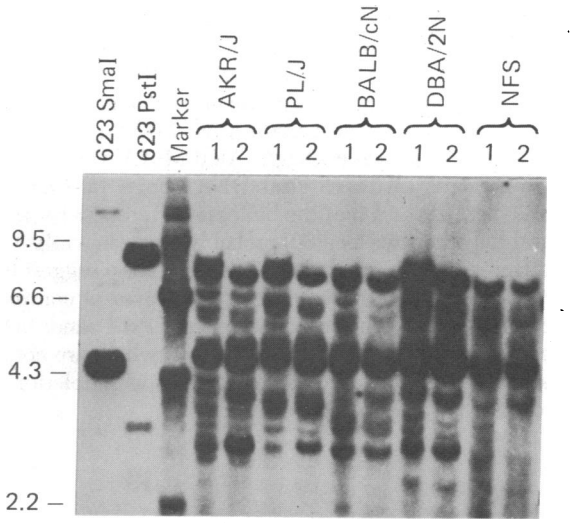

FIG. 5. Hybridization of *Pst* I- and *Pst* I/*Hin*dIII-digested mouse embryo DNAs. Cell DNAs were electrophoresed in a 0.5% agarose gel, transferred to nitrocellulose filter, and hybridized with the ^{32}P-labeled 5′ viral probe. Lanes: 1, *Pst* I-digested DNA; 2, *Pst* I/*Hin*dIII-digested DNAs. Marker DNAs were as in Fig. 3.

DISCUSSION

In this study, we began to investigate the structure of endogenous MuLV DNAs in normal murine cells. This analysis has been facilitated by developing physical maps for MuLVs isolated from these mouse strains and by the availability of rigidly defined viral probes derived from molecularly cloned viral DNA. We have identified a probe specific for ecotropic viruses as well as restriction fragments in cell DNA which are specific for ecotropic and xenotropic MuLVs. These studies should ultimately provide a correlation between the structure of endogenous viral DNAs and the viruses isolated from these mice. This knowledge is also necessary as a baseline for determining whether a newly isolated virus represents an endogenous viral genome or has arisen as a result of recombination (28).

It should be noted that any MuLV shares extensive sequence homology with the MuLVs of other classes (31). However, previous studies have shown that cells from high-ecotropic mice contained multiple genome equivalents of ecotropic viral DNA, whereas low-ecotropic mice contained only one complete copy (2, 10–14). The ecotropic type-specific probe described in this report has confirmed this result for several mouse strains and has enabled us to identify ecotropic-specific bands in restriction endonuclease-digested cellular DNAs, which should simplify genetic studies involving these genomes. For different strains, the ecotropic-specific bands in the *Eco*RI-digested cell DNAs were of different lengths, which is consistent with the hypothesis that these viral DNAs were introduced into the cell genome of each strain as a recent independent integration event. Although these viral genomes may be relatively stable, heterogeneity of viral integration sites may occur even within the same strain by excision or insertion of viral DNA, as implied by the differences noted between AKR/J and AKR/N DNA. These differences have also been noted by others (14).

It has previously been shown that ecotropic virus inducing loci contain some structural viral DNA sequences (10–14). The hybridization of restriction endonuclease-digested cell DNA with probes from defined regions of the viral genome has led us to conclude that these loci contain a virtually complete linear copy of the viral DNA with sequences from the LTR at each end. For example, all three ecotropic-specific *Eco*RI bands in AKR cell DNA are digested to a single 8.2-kbp *Pst* I band; this result correlates with *Pst* I digestion yielding an 8.2-kbp internal viral fragment in MuLV isolated from mice congeneic for *Akv-1* and *Akv-2* (unpublished data). In addition to this 8.2-kbp *Pst* I band's hybridizing to the ecotropic-specific probe and to probes from the other segments of the virus, it was digested by *Hin*dIII (in contrast to the presumptive 7.6-kbp xenotropic band), which also cleaves ecotropic AKR MuLV DNA. These results imply that all three viral DNA copies in AKR are structurally similar and that the biologic differences between *Akv-3* and the other two loci (30) probably reside in a relatively subtle difference in this molecule. Our results also suggest that some endogenous xenotropic viral DNAs represent complete viral genomes as well. The ecotropic-specific *Pst* I bands in the high ecotropic strains and *M. m. castaneus* which are not 8.2 kbp may represent ecotropic genomes either with deletions or with a different *Pst* I-digestion pattern.

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